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GENETIC COMPLEMENTATION TO IDENTIFY DNA ELEMENTS THAT INFLUENCE COMPLEMENT RESISTANCE IN *LEISHMANIA CHAGASI*

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ABSTRACT: Past studies showed that *Leishmania* spp. promastigotes exhibit differential sensitivity to complement mediated lysis (CML) during development in vitro and in vivo. *Leishmania chagasi* promastigotes in cultures during logarithmic and stationary growth phases are CML-sensitive or CML-resistant when exposed to human serum, respectively, but only in cultures recently initiated with parasites from infected animals; serially passaged cultures become constitutively CML-sensitive regardless of growth phase. Building on these observations, a genetic screen was conducted to identify novel complement resistance factors of *L. chagasi*. A cosmid library containing genomic DNA was transfected into a promastigote line previously subjected to >50 serial passages. Selection with human serum for CML resistance yielded 12 transfectant clones. Cosmids isolated from 7 of these clones conferred CML resistance when transfected into an independent, high-passage promastigote culture; at 12% human serum, the mean survival of transfectants was 37% (±11.6%), and that of control transfectants was about 1%. Inserts within the 7 cosmids were unique. Determination of the complete DNA sequence for 1 cosmid indicated that its 32-kilobase insert was 89% identical (overall) to a 31-kilobase region of *Leishmania major* chromosome 36, which is predicted to encode 6 genes, all of which encode hypothetical proteins.

Leishmania spp. (Trypanosomatidae) are the etiologic agents of leishmaniasis, a disease group that ranges in severity from self-healing cutaneous lesions to potentially fatal visceral infections. These protozoan parasites alternate between an intracellular amastigote form located within phagocytic cells of the vertebrate host immune system, and a promastigote form located in the midgut of the sandfly vector. After a female fly feeds on the blood of an infected host, amastigotes within ingested phagocytic cells are released and quickly differentiate into procyclic promastigotes that attach to the midgut wall. Over a period of 1 wk to several weeks within the fly, promastigote development is characterized by a progression through a series of intermediate parasite stages that are distinguishable by location, morphology, and replicative/nonreplicative status (Rogers et al., 2002), a progression that yields the final stage, the metacyclic promastigote. Metacyclic promastigotes migrate to the anterior gut, where they become available for inoculation into a mammalian host when the sandfly next takes a meal of blood (reviewed in Schlein, 1993). Metacyclic promastigotes are distinguishable from procyclic promastigotes based on their morphology; high infectivity to vertebrates (Sacks and Perkins, 1984; Schlein, 1993; Saraiva et al., 1995); increased or differential outer surface glycosylation state, including lipophosphoglycan, or LPG (Sacks et al., 1985, 1995); and increased expression of surface glycoproteins, major surface protease (MSP, also known as GP63 [Wilson et al., 1993]), and promastigote surface antigen (PSA, also known as GP46 [Beetham et al., 1997]). Importantly, these properties of procyclic and metacyclic promastigotes are mirrored when parasites that have been recently isolated from parasitized vertebrate hosts are grown in axenic cultures as promastigotes; procyclic and metacyclic promastigotes are found in cultures during logarithmic and stationary growth phases, respectively (Sacks and Perkins, 1984; Zarley et al., 1991).

Another trait of metacyclic promastigotes of all *Leishmania* spp. examined is resistance to the complement component of the vertebrate immune system (Franke et al., 1985; Joshi et al., 1998; Noronha et al., 1998; Pinto-da-Silva et al., 2002). The host complement system is one of the first antimicrobial immune mechanisms encountered by newly inoculated metacyclic promastigotes. Complement consists of multiple serum proteins having the potential to bind in series to invading microbial pathogens. Complement recognition/binding can kill microbes by either of 2 mechanisms. In one, complement proteins bound to microbes function as opsonins that facilitate receptor-mediated uptake by phagocytic cells. In the other, completion of the complement cascade results in formation of a membrane attack complex on the microbial cell surface that induces cell lysis by disrupting the membrane integrity, a process called complement-mediated lysis (CML).

Metacyclic promastigotes utilize complement opsonization to facilitate their receptor-mediated entry into phagocytic cells within which the parasites survive and replicate during their amastigote life stage (Blackwell et al., 1985; Mosser and Edelson, 1985; Da Silva et al., 1989; Puentes et al., 1990; Dominguez and Torano, 1999). To effectively use complement toward gaining entry into phagocytes, metacyclic promastigotes must also avoid CML. Past studies have shown that metacyclic promastigotes do avoid CML, and that this property may involve 3 highly abundant surface macromolecules: MSP, LPG, and PSA. MSP, a zinc protease, is believed to block the complement cascade by MSP-induced proteolysis of complement factor C3b to an iC3b-like form that retains opsonin function while losing additional activities required for assembly of the membrane attack complex (Brittingham et al., 1995). LPG-induced protection to CML is associated with a lengthening of LPG or a change in terminal glycosylation residues (or both), and these changes may act by preventing proper localization of membrane attack complex, relative to the parasite surface membrane (Puentes et al., 1990). Restored expression of PSA in PSA-minus high-passage promastigotes resulted in increased resistance to serum lysis (Lincoln et al., 2004); the mechanism for this effect has not been determined.

Pathogens have evolved multiple mechanisms with which to evade the antimicrobial effects of complement activation, and frequently, individual species (including, as noted above, of

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Leishmania spp.) have several complement-evasion strategies that can operate simultaneously and independently (Wurzner, 1999). One approach that, to our knowledge, has not previously been used to identify novel molecules involved in microbial resistance to CML is that of genetic complementation. Recent studies determined that serial passage of *Leishmania chagasi* causes promastigotes to become constitutively sensitive to CML regardless of culture growth state (Lincoln et al., 2004). Experiments were, therefore, undertaken that aimed, by genetic complementation of serially passaged cells, to identify genetic elements that function in CML-resistance in *L. chagasi*.

MATERIALS AND METHODS

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis) were maintained in Golden Syrian hamsters by serial passage as described (Pearson and Steigbigel, 1980). Amastigotes were differentiated into promastigotes, and the promastigotes were subsequently cultured using supplemented modified minimum essential media (HOMEM) as previously described (Ramamoorthy et al., 1992). Promastigote cultures seeded at 1.0×10^6 cells/ml were split to 1.0×10^6 cells/ml 7 days later, a time that corresponded to 2–3 days after reaching stationary growth phase with a density of $2–4 \times 10^7$ cells/ml. Logarithmic and stationary phases of cultures were determined by cell morphology and culture density as described (Zarley et al., 1991). Cell cultures were considered low-passage (LP) if serially passaged for ≤3 wk. High-passage cells (HP) used in the following experiments were passaged for >50 wk.

Human serum

Human serum was derived from the pooled extracts of multiple donors. Blood drawn from naive human donors into serum separator tubes was incubated for 1 hr at 23 C, then 1.5 hr at 0 C, and then centrifuged at 450 *g* for 10 min at 4 C. Resulting serum fractions were pooled, aliquoted, stored at −80 C, and thawed on ice when needed for assays.

Complement assays

Promastigotes were pelleted by centrifugation at 1,000 *g* for 5 min, washed in phosphate-buffered saline (PBS pH 7.8), then resuspended in PBS at 7×10^7 cells/ml. Cell aliquots (50 μl) in 96-well plates were brought to 100 μl total volume with PBS, or human serum, or both, then incubated at 37 C for 30 min. Cells were then diluted 1:10 in 0 C PBS, and motile cells were counted on a hemocytometer. At high serum concentrations, nonviable, CML-sensitive cells are observed primarily as cell debris, whereas at lower serum concentrations CML-sensitive cells often are still visible, but appear as nonmotile and granular (i.e., ghost cells) that were not counted as viable. The percentage of cell survival was calculated as the ratio of motile cells present in incubations with serum versus without serum.

Library construction

Total genomic DNA isolated with a DNAzol kit (Invitrogen, Carlsbad, California) from $2–3 \times 10^9$ LP promastigotes was partially digested with restriction endonuclease *Sau*3A to yield DNA fragments of predominantly 20–40 kb (as determined by ethidium bromide staining of electrohporetically separated digest products). Digested DNA was ligated into *Bam*HI-digested cosmid vector cLHYG (a kind gift from S. Beverley), then packaged into viral particles and amplified in *Escherichia coli* by kit (Gigapack III XL; Stratagene, La Jolla, California). Phage-infected bacterial colonies were pooled (3 pools, ~2,700 colonies per pool); individual pools were amplified in *E. coli*, and then cosmid DNA was isolated (Maxi Kit; Qiagen, Valencia, California) for use in parasite transformation experiments.

Transfection and selection of parasites

Transfection of HP promastigotes by electroporation of cosmid DNA was largely as described (Kapler et al., 1990), except that electroporation occurred in 75% electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose) and 25% CytoMix (120 mM KCl 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, and 5 mM $MgCl_2$). Electroporation in 0.4-cm cuvettes used the following electrical constraints: 2 kV and 25 μfd. High-passage promastigote cultures in late logarithmic growth phase ($0.8–1.0 \times 10^7$ cells/ml) were transfected using 8×10^7 cells per transfection reaction. Following transfection, cells were incubated for 72 hr in 1× M199 media (Cellgro, Herndon, Virginia) to allow recovery. Cells were pelleted by centrifugation for 5 min at 1,000 *g*, resuspended in HOMEM containing 25% pooled human serum (see *Human Serum*), and incubated at 37 C for 30 min. Cells were again pelleted, the pellet was washed in PBS, resuspended in 500 μl of HOMEM, then spread onto semisolid media containing M199 medium (Invitrogen) supplemented with 100 μg/ml hygromycin (Sigma, St. Louis, Missouri). Colonies were isolated from plates 2–3 wk later, and then individual isolates were cultured in HOMEM supplemented with 10% heat-inactivated fetal calf serum and 100 μg/ml hygromycin.

Cosmid DNA was recovered from transfected promastigotes using a modified alkaline lysis procedure. Promastigotes from stationary-phase cultures (2×10^8 cells) were pelleted at 1,500 *g* for 5 min, resuspended in TGE buffer (25 mM Tris pH 8.0, 10 mM EDTA, and 50 mM glucose), and lysed in 200 μl of 0.2 M NaOH:1% SDS. Following 5 min of incubation on ice, the protein was precipitated by adding 150 μl of sodium acetate pH 4.8, then centrifuged at 14,000 *g* for 5 min. The resulting supernatant was extracted (with phenol:chloroform:isoamyl alcohol, 25:24:1), and the DNA within the aqueous phase was precipitated by the addition of 2 volumes of ethanol and centrifugation (5 min, 14,000 *g*). Precipitated DNA was resuspended in 200 μl of buffer (25 mM Tris pH 8.0, 10 mM EDTA pH 8.0), then reprecipitated by bringing the solution to 6.5% in polyethylene glycol (weight by volume) and 0.4 M in NaCl, incubated on ice for 1 hr, and pelleted by centrifugation (5 min, 14,000 *g*). The resulting pellet was washed in 70% ethanol, then resuspended in 20 μl of buffer (25 mM Tris pH 8.0, 10 mM EDTA pH 8.0).

Sequencing and annotation

To sequence the entire insert of cosmid #2, cosmid DNA fragments resulting from *Sac*I digestion were cloned into the *Sac*I site of plasmid *pBluescript* SK(−) (Stratagene), or were subjected to religation. Initial sequencing reactions used universal primers that annealed to the plasmid sequence flanking the inserts; subsequent reactions used primers that annealed to the sequence that had been determined in the previous sequencing reaction. Sequences from the subclones were aligned and connected by directly sequencing cosmid template using outward-facing primers designed against the sequence of the ends of the subclone inserts. Sequencing of the ends of the inserts within cosmids #1 and #3–6 used primers that annealed to cosmid vector sequence flanking the inserts. Cosmid insert sequences were analyzed for predicted coding sequences and for similarity to the *L. major* genome (at http://www.genedb.org, Sanger Institute, Cambridge, U.K.) using several software programs: GLIMMER and Artemis (with the assistance of Al Ivans, The Sanger Institute, Cambridge, U.K.), BLAST, GAP, and BestFit.

RESULTS

Only *L. chagasi* parasites within low-passage stationary-phase cultures resist CML

Complement sensitivity assays using human serum were performed on promastigotes taken from LP and HP cultures at logarithmic and stationary growth phases. HP cells were CML-sensitive in cultures during both logarithmic and stationary growth phases (LC_{50} = 4.7% and 4.8% serum, respectively; Fig. 1b). In comparison, LP cells exhibited a differential CML sensitivity that was dependant on the culture growth phase (Fig. 1a). Cells from LP cultures during logarithmic growth phase were highly sensitive to CML (LC_{50} = 4.5% serum), whereas cells from stationary-phase cultures were highly resistant, with

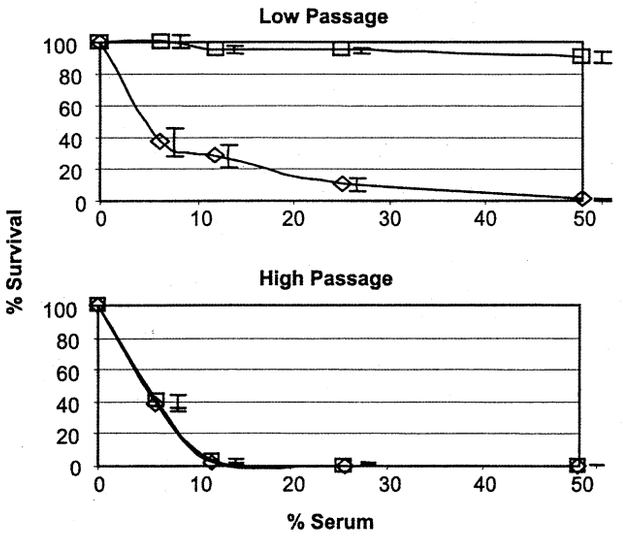

FIGURE 1. Promastigote resistance to complement-mediated lysis. After passage in culture for less than 5 wk (**a**) or >50 wk (**b**), promastigotes from cultures at logarithmic (◊) and stationary (□) growth phase were incubated for 30 min at 37 C in human serum at 0, 6, 12.5, 25, and 50%. The percentage of cell survival was calculated as the ratio of motile cells present after incubation with serum versus without serum (see *Materials and Methods*). Error bars indicate the SEM of 3 experiments.

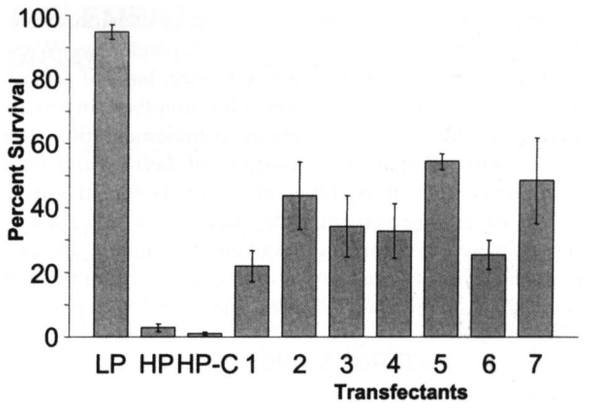

FIGURE 2. Cosmid-transformed promastigote resistance to complement-mediated lysis. Sensitivity of promastigotes incubated in 12.5% serum to CML was assayed. Stationary-phase promastigotes included nontransfected low-passage (LP) and high-passage (HP) cells, and HP cells transfected with an irrelevant cosmid (HP-C) or with cosmid isolated from previous transfectants selected for CML-resistant phenotype (#1–7). The mean survival of the serum-selected transformants was 37% (± 11.6% at 99% confidence interval): negative controls were 2.0 ± 1.1% at 95% confidence interval; error bars indicate the SEM of 3 to 5 experiments.

nearly 100% surviving even at the highest serum concentrations; consequently, the LC_{50} could not be calculated/extrapolated for these CML-resistant cells.

To screen for genetic elements able to influence CML sensitivity in promastigotes from HP cultures, a genomic complementation library was constructed using the cosmid vector cLHYG; DNA elements contained within this vector allow cosmid replication and drug resistance selection in *Leishmania* spp. and in *E. coli* (Ryan et al., 1993). Partial restriction enzyme digestion (using *Sau*3A) of *L. chagasi* genomic DNA yielded 20–50 kb DNA fragments that were ligated into cosmid c-LHYG. Following transfection of *E. coli* with packaged cosmid, the insert size of 20 randomly selected bacterial cosmid clones was determined to range between 22 to 40 kb (mean = 35 kb).

Identification of 6 cosmid-transfected high-passage clones having increased resistance to CML

To obtain a heterogeneous pool of cosmids, bacterial colonies containing the library clones were pooled (3 pools, each with 2,700 colonies per pool), amplified, and then their cosmid DNA was isolated. The resulting pooled cosmid DNA, representative of 8,700 colonies, was transfected by electroporation into CML-sensitive, high-passage promastigotes from the same culture line as was used for the CML assays reported in Figure 1. To select for CML-resistant parasites, promastigotes from HP cultures in late logarithmic growth phase were transfected by electroporation (see *Materials and Methods*) and allowed to recover in 1× M199 for 48 hr. Transfectants were concentrated by centrifugation and resuspended in PBS containing 25% naive human serum for 30 min. Surviving transfectants were clonally isolated on semisolid medium and then expanded in liquid medium (see *Materials and Methods*). Assay of the clonal isolates for CML sensitivity identified 20 clones that were CML-resistant, of which 12 were selected for additional analysis.

Restored CML resistance is associated with the cosmid construct

Based on their CML-resistant phenotype, clones were analyzed to confirm that the CML resistance was due to cosmid insert DNA. Initially, cosmids were successfully recovered/isolated from 8 of 12 CML-resistant clones (see *Materials and Methods*). Two of these 8 cosmids were determined to contain identical inserts (in experiments described later in this paper); subsequent experiments described herein pertain to the remaining 7 cosmids only. The 7 purified cosmids were then transfected back into CML-sensitive promastigotes (i.e., promastigotes from HP cultures), and resulting transfectants were clonally isolated and assayed for CML sensitivity. In all cases, the CML-resistant phenotype followed the cosmid constructs; each transfectant clone exhibited CML resistance when exposed to 12.5% serum. The survival of individual clones ranged from a mean of 22% to 54%, and the combined average survival among all clones was 37% ± 11.6% at a 99% confidence interval (Fig. 2). The CML resistance of these clones was not due to the cLHYG backbone within the cosmid constructs; control transfectants containing cLHYG with an irrelevant insert were as CML-sensitive as the nontransfected, HP cells (i.e., column HP-C [Fig. 2]). The positive control included in all experiments (LP promastigotes from stationary-phase cultures) always yielded survival rates of nearly 100% at all serum concentrations tested. When the 6 clonal transfectants were assayed at higher serum concentrations (25% and up), survival was indistinguishable from that of the HP control transfectants and HP nontransformed cells.

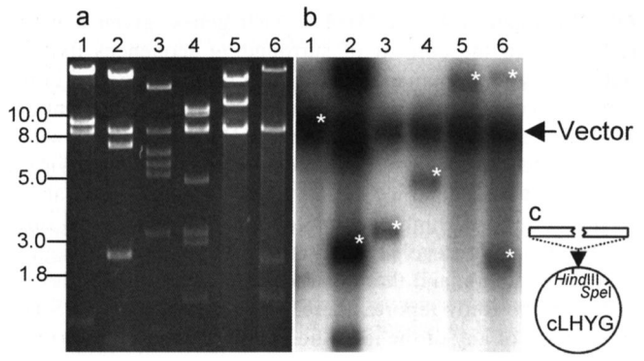

FIGURE 3. Diversity among cosmid inserts. (**a**) Cosmid DNA from serum-resistant high-passage clones #1–6 was digested with *Spe*I and *Hin*dIII, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining. (**b**) The DNA within the gel was transferred to nylon membrane, probed with radiolabeled DNA of cosmid #2 (previously digested with *Spe*I and *Hin*dIII) and then visualized by autoradiography; bands composed completely or partially of vector are labeled as Vector or with an asterisk (*), respectively. (**c**) A diagram of the cLHYG cosmid with an insert demonstrates the position of the vector *Spe*I and *Hin*dIII restriction enzyme recognition sites relative to the insert.

Each of the isolated CML-resistant cosmids contains a unique insert

As an indicator of the heterogeneity between the inserts of the 7 cosmids, restriction fragment-length polymorphism analysis and Southern hybridization experiments determined that each cosmid contained a unique insert. A representative experiment is shown (Fig. 3) in which 6 cosmids were digested with *Spe*I-*Hin*dIII, separated by agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 3a). *Hin*dIII cuts within the cosmid backbone at a single site located near one side of the insert site, and *Spe*I cuts the vector 8.4 kb from the *Hin*dIII site, which is about 1.8 kb from the other side of the insertion site (as diagramed in Fig. 3c). *Spe*I-*Hin*dIII digestion of all CML-resistant cosmids yielded differing DNA banding patterns, although all contained the expected 8.4-kb band corresponding to the *Spe*I-*Hin*dIII fragment of the cLHYG vector (Fig. 3). To assess whether regions of high identity were present among the inserts, Southern analysis was performed using radiolabeled DNA from an individual cosmid clone to probe the DNA from all clones. Using cosmid #2 DNA as a probe (Fig. 3b), more than 6 bands were detected among the digest products of cosmid #2, whereas only 2 bands (corresponding to the 8.4-kb vector fragment and a variably sized vector-containing fragment) were detected in the other CML-resistant cosmid clones. The variable-sized band corresponding to the 1.8-kb fragment of vector is lengthened in proportion to the location, within the insert, of the *Spe*I or *Hin*dIII site nearest the vector. In addition, cosmid #6 contained a third band (2.9 kb) that hybridized to cosmid #2 probe. This third band is most likely due to rearrangements within the cosmid vector backbone, because the same band of cosmid #6 was also detected with radiolabeled probes made from the DNA of each of the other cosmids (data not shown). Additionally, when cosmid #6 was labeled as probe, no new bands were detected in the digests from cosmids #1–5 (data not shown). Equivalent experiments were performed using the same digestion and labeling procedure as for cosmid #2, but using cosmid #1 and #3–6 as probes (data not shown). These experiments indicated that each of the 6 CML-resistant cosmids contains a unique insert and that the insert sizes vary between 19 and 33 kb.

Sequencing of cosmid #2 indicates identity to *L. major* chromosome #36

Cosmid #2 was selected for complete sequence determination based on initial experiments in which transfectants bearing cosmid #2 had the highest levels of CML resistance among the isolated clones. BLAST comparison of the 31.8-kb insert sequence (GenBank AY656839) to that of the *L. major* genome indicates that the insert is homologous to a 29.9-kb region of *L. major* chromosome 36, based on the high sequence identity that exists between these sequences (BLAST scores >1,000, smallest sum probability $<1.0e^{-50}$) and on the lack of high overall identity between cosmid #2 to any other regions of the *L. major* genome. A graphical representation of the aligned regions of cosmid #2 and *L. major* demonstrates the high synteny that exists between these *L. major* and *L. chagasi* sequences (Fig. 4). The insert of cosmid #2 is predicted by analysis with GLIMMER (see *Materials and Methods*) to code for the same 6 genes/proteins that are predicted for the 29.9-kb segment of *L. major* (Fig. 4; labeled A–F and 0790–0840, respectively). Comparison of the sequence of *L. chagasi* cosmid #2 insert and that of *L. major* chromosome 36 by GAP or BestFit programs indicates an identity of 90–94% for the predicted coding sequences when compared over their full length (Fig. 4), and 84–87% for noncoding sequences (data not shown). Queries of gene and protein databases using BLAST and PSI-BLAST searches with the predicted genes/proteins of cosmid #2 failed to identify any similarity to proteins whose activities could perturb complement lysis (i.e., proteases). Two of the genes, B and C (Fig. 4), have predicted signal peptide sequences, indicating possible uptake to the endoplasmic reticulum, but none of the predicted proteins contain either a transmembrane spanning domain or a hydrophobic carboxy-terminus sequence that (respectively) would be typical for membrane-spanning or glycosylphosphatidylinositol-anchored outer surface proteins. Gene

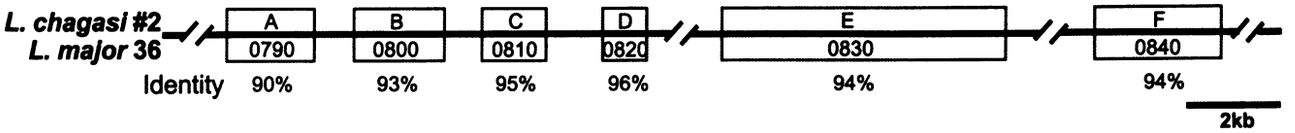

FIGURE 4. *Leishmania chagasi* cosmid #2 insert and homologous region of *L. major* chromosome 36. The predicted coding sequences (boxes), noncoding sequences (line between boxes), and the percentage identity shared between homologous coding sequences are as indicated. Numbers for *L. major* coding sequences (i.e., 0790), correspond to chromosome 36 coding sequence identifiers assigned by the *L. major* genome project (see *Materials and Methods*).

B has homology to an mRNA basal transcription factor subunit p52 in mice and gene D to an ADP-ribosylation factor in mice, *Candida albicans* and *Caenorhabditis elegans*; BLAST scores for identity of these genes to B and D were less than 6×10^{-5}. Blast and PSI-Blast analyses of genes A, C, E, and F failed to indicate a high level of identity to any other sequences having known or predicted functions or activities.

DISCUSSION

These experiments demonstrate that the technique of gene complementation can be used with HP cells that are CML sensitive to identify genetic elements that increase *Leishmania* spp. resistance to CML. A better understanding of the phenomenological data will require a mechanistic understanding of how DNA of the cosmid inserts acts to confer resistance to CML. Possible mechanisms include blocking the complement cascade to prevent assembly of functional membrane attack complex, shedding complement components bound to the parasite surface, and increasing the tolerance of the parasite membrane surface for assembled membrane attack complexes. Regardless of the mechanism by which resistance to complement lysis is achieved, it will also be important to determine whether gene products of the cosmids act directly to mediate the resistance, or whether they act indirectly (i.e., by modulating levels of surface molecules that act directly to confer complement resistance). Previous studies have identified 2 surface molecules expressed in high abundance on CML-resistant cells, MSP and LPG, which appear to function in resistance to lysis by complement. MSP, a zinc protease, is believed to interrupt the complement cascade by cleaving the C3b component to an inactive form that does not support assembly of a functional membrane attack complex. LPG undergoes lengthening and changes in terminal glycosylation state, which may prevent interaction of functional membrane attack complexes with the membrane surface.

In addition to mechanistic studies, further support of a biological role for these cosmids will be supplied when individual genes that are present within the cosmid insert are characterized as contributing to CML resistance. The sequencing of cosmid clone #2 constitutes an important step toward the identification of functional genes that are present within the cosmid insert, and will enable the construction of a series of deletion or expression subclones (or both) to be used in transfection studies to identify those insert regions that are important for the CML resistance conferred by the parent cosmid construct.

One potential limitation to the use of gene complementation to identify genetic elements involved in CML resistance is the possibility that this, or any episome-based, methodology will lend itself to the identification of only a subset of CML-resistance genes. Specifically, the technique may not be effective in identifying certain genes whose chromosomal regulation is mirrored when expressed in episomal systems. For example, both PSA and MSPS are developmentally regulated genes whose increased abundance in metacyclic cells is due to posttranscriptional regulation of mRNA abundance. Episomal expression of PSA and MSPS appears to model normal expression of the chromosomally located genes in studies that have successfully used episome expression systems to identify *cis*-acting elements involved in the regulated expression of genes (Myung et al., 2002; Beetham et al., 2003). For such genes, given that they are present in the context of surrounding sequences likely to contain *cis*-acting elements involved in regulated expression, it seems unlikely that the technique of episomal complementation will result in expression of the episomal genes in growth or culture states in which the chromosomal copies are down-regulated. However, contrary to this supposition, there is always the possibility that high episome copy numbers per cell might result in transcript levels that titrate out the activity of negative regulatory factors, and thereby allow higher expression levels). In fact, in the study reported here, neither PSA nor MSPS were components of any of the identified cosmids (as was determined by Southern analysis; data not shown).

Importantly, not all developmentally regulated genes utilize a system for regulated expression that is successfully modeled in episome-based systems. For instance, MSPL, a form of MSP that is up-regulated within procyclic promastigotes within cultures at logarithmic growth phase, undergoes modulated expression via posttranscriptional regulation of mRNA abundance, but this expression has not been found to be reproducible in episome-based systems (Donelson, 1995). Perhaps genes that are regulated in like fashion to that of MSPL, and not that of PSA or MSPS, will constitute the subset of CML-resistance genes that are amenable to complementation using an episome-based system.

We observed that none of the cosmid clones selected here restored CML resistance to the level found in cells from low-passage cultures at stationary growth phase. One possible explanation for this is that the product of the CML resistance gene located within the cosmid insert may, for full activity, require other factors that are down-regulated in HP cells; therefore, episomal reexpression of this single gene would not confer absolute CML resistance. Such factors could include MSP, metacyclic-LPG, and PSA; molecules identified in studies as influencing parasite resistance to complement lysis (Puentes et al., 1990; Brittingham et al., 1995; Lincoln et al., 2004). Another possible explanation is that the cosmid-encoded genes having CML resistance function may be expressed at lower levels in the serially passaged transformed cells than are the corresponding chromosome-located genes in LP cells.

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